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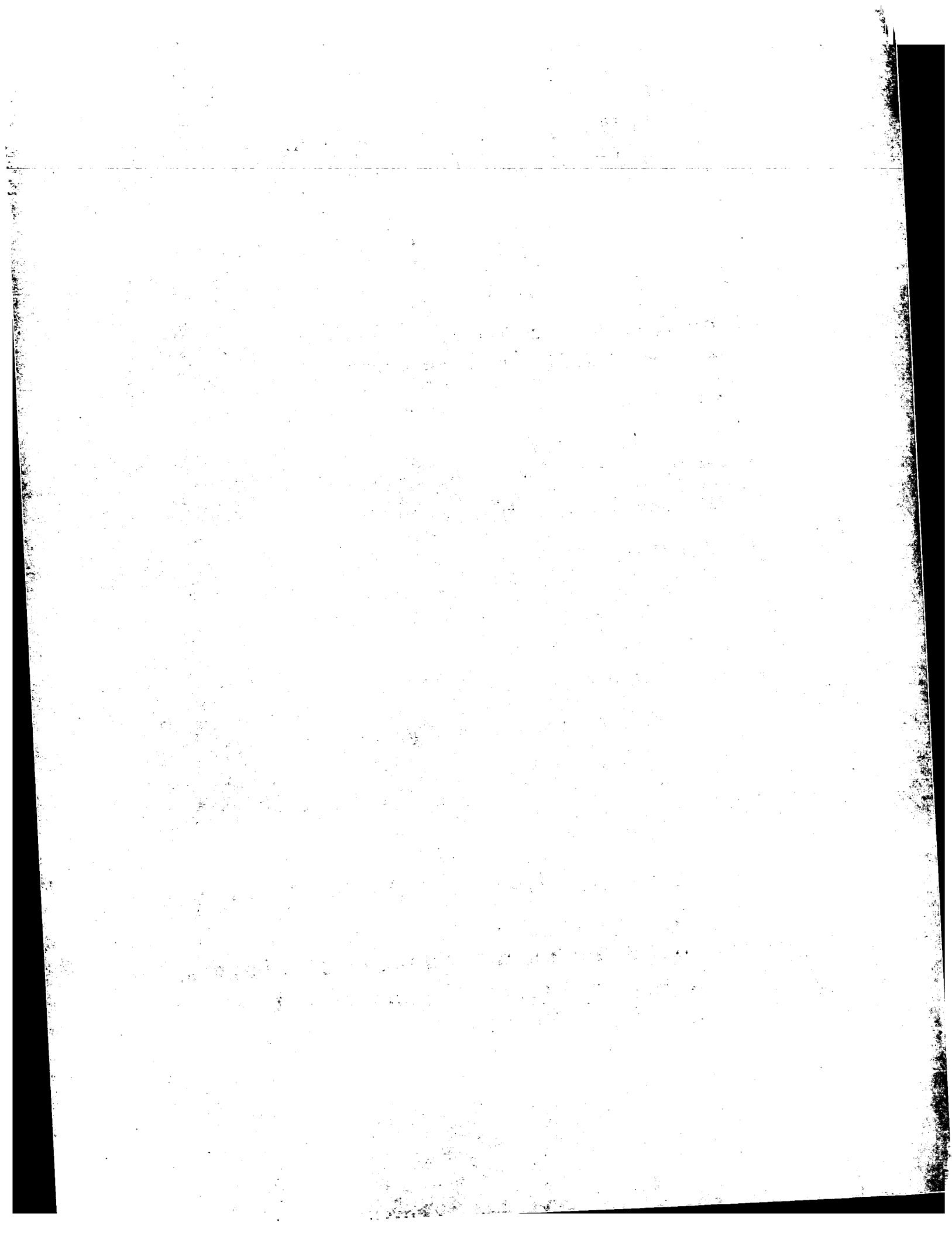
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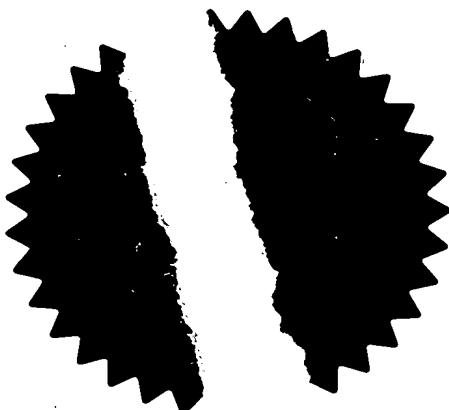
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GB 0104696.0

By virtue of a direction given under Section 30 of the Patents Act 1977, the application is proceeding in the name of:

HEALTH PROTECTION AGENCY,
Porton Down,
SALISBURY,
Wiltshire,
SP4 0JG,
United Kingdom

Incorporated in the United Kingdom,

[ADP No. 08638108001]



1/77

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2. Patent application number (The Patent Office will fill in this part)	0104696.0		
3. Full name, address and postcode of the or of each applicant (underline all surnames)	Microbiological Research Authority CAMR Porton Down Salisbury Wiltshire SP4 0JG <i>SECTION 30 (1977 ACT) APPLICATION FILED 12/8/03</i> GB C6566160001		
Patents ADP number (if you know it)			
If the applicant is a corporate body, give the country/state of its incorporation			
4. Title of the invention	Degradation of TSE and similar agents		
5. Name of your agent (if you have one)	MATHYS & SQUIRE 100 Gray's Inn Road London WC1X 8AL United Kingdom		
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**DUP
LICATE**

DEGRADATION OF TSE AND SIMILAR AGENTS

5 The invention is in the field of methods and compositions for the sterilisation of materials and apparatus that may have been contaminated with infectious agents. In particular, the invention relates to methods for the inactivation of transmissible spongiform encephalopathy (TSE) agents and provides compositions for degrading TSE located on or within infected materials.

10 Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurological diseases that include Creutzfeld-Jacob disease (CJD) and Kuru in humans, bovine spongiform encephalopathy (BSE) in cattle and Scrapie in sheep. TSEs are characterised by the conversion of a normal host protein into a pathogenic protein within the brain tissue of an infected animal. The pathogenic form of protein is often referred to as a prion and is highly resistant to physical and chemical degradation. The prion is believed to be the 15 transmissible agent through which the TSE disease is passed on between animals.

20 There has been considerable public alarm in recent years over the risks associated with consumption of meat products, and especially beef, potentially infected with BSE, the bovine form of TSE. Much of this concern is associated with the belief that the BSE prion when eaten by a human may in some cases cause the incurable human form of the disease, referred to as variant CJD (vCJD). Rigorous practices have been adopted in the agricultural 25 and meat rendering industries to reduce the risk of cross contamination between BSE infected carcasses and meat that is destined for human consumption or other animal derived products such as tallow. However, BSE infected animals can still be unintentionally processed in abattoirs, especially if the animal is in the early stages of the disease and therefore undetected as 30 an infected TSE host. There is also a considerable risk in disposal of known BSE infected material, particularly if the equipment used in the disposal operation is then reused in normal rendering practices without adequate sterilisation.

35 Sterilisation of instruments and equipment following potential exposure to TSE infected tissue is of primary importance. In particular, surgical equipment such as scalpels, forceps and endoscopes should be thoroughly sterilised before use on patients to avoid disease transmission.

It has been reported that the CJD infectious agent was accidentally transferred on surgical electrodes, inserted into the brain of a human patient with CJD, to two other previously uninfected patients (Bernouli et al (1977) The Lancet i: pp478-479). The electrodes concerned were sterilised with ethanol and formaldehyde vapour between each procedure, conditions previously thought to be sufficient to eliminate virtually all infectious agents, and yet the CJD infectious agent was able to withstand such harsh conditions and infect the recipient patients' brain tissue.

TSE transmission is typically observed in cases where infected material is transferred between animals or implanted into an animal. As described previously, incomplete or inadequate sterilisation of surgical instruments can lead to such transfer of infected material between patients. Even the most rigorous chemical cleaning and steam sterilisation procedures can fail to remove blood and tissue from surgical instruments, especially in the jaws or joints of forceps and clamps (Laurenson (1999) The Lancet, 20 Nov). Thus, the risk of unintentional TSE transfer can be unnecessarily high.

TSE agents, or prions, are known to be highly resistant to denaturation and degradation, more so than would normally be expected for a protein. Taylor (J. Hosp. Infect. (1999) 43 supplement, pp S69-76) reviews a number of methods for inactivating prion proteins.

Chemical methods for inactivating TSE prion proteins include treatment of infected material with sodium hydroxide or sodium hypochlorite solutions (Taylor et al. (1994) Arch. Virol. 139, pp313-326), although, infectivity of the prion is shown to survive exposure to 2M sodium hydroxide for up to two hours.

Alternative methods for inactivating TSE prions include autoclaving, but again BSE and scrapie agent has been shown to survive treatment at 134-138°C for 18 minutes (Taylor et al. ibid). Thus, a combined chemical/heating approach has been proposed in which infected materials are exposed to 1M sodium hydroxide followed by autoclaving at 121 °C for between 30 and 60 minutes (Taylor, J. Hosp. Infect. (1999) 43 supplement, pp S69-76). This combined method has shown that inactivation of TSE infectious agents can be achieved, albeit under very harsh conditions.

However, many materials, such as plastics, polymers and non-protein animal derivatives, cannot be exposed to such extreme conditions without themselves being destroyed. The chemical and physical processes described above are only really suitable for sterilising metal instruments and surgical tools that are not too large in size and which can fit inside a standard autoclave. More delicate instruments such as endoscopes cannot be exposed to extreme conditions of high temperature without the risk of permanent damage to their internal components.

Further, chemical processes typically involve the use of caustic and/or chaotropic agents which are hazardous to handle and dispose of. It would, therefore, be desirable to provide a method for inactivating TSE prions without the need to use large amounts of hazardous substances and which method could be scaled up for use on larger objects and areas as well as on smaller objects.

Taylor (Vet. J. (2000) 159 pp10-17) describes tests using proteolytic enzymes to deactivate prion proteins. Proteases such as trypsin have little effect in non-denaturing conditions (Taylor (2000) p. 14) but other proteases such as proteinase K may have an effect on TSE infectivity following prolonged digestion times. However, the majority of current TSE inactivation methods are aimed towards chemical and physical degradation procedures.

It is an object of the invention, therefore, to provide methods and means for effectively inactivating TSE infectious agents under conditions that can be readily applied to a variety of locations and situations. It is a further object of the invention to reduce the need for extreme conditions of very high temperature and harsh chemical denaturants in order to inactivate TSE agents located on or within TSE infected materials.

A first aspect of the invention provides a method for inactivating a TSE agent comprising exposing the TSE agent to a thermostable proteolytic enzyme.

The term transmissible spongiform encephalopathy (TSE) agent is intended to encompass all neurological diseases that are apparently transmitted via a pathogenic prion protein intermediate. Such TSEs typically include the human diseases Creutzfeld-Jacob disease (CJD), variant Creutzfeld-Jacob disease

5 (vCJD), Kuru, fatal familial insomnia and Gerstmann-Straussler-Scheinker syndrome. Non-human TSEs include bovine spongiform encephalopathy (BSE), scrapie, feline spongiform encephalopathy, chronic wasting disease, and transmissible mink encephalopathy. Given that vCJD is currently understood to be a human form of BSE, it is apparent that certain TSE agents are capable of crossing the species barrier and that novel TSEs from non-bovine sources could become evident in future.

10 The methods and compositions of the invention are suitable for the inactivation of TSE agent in apparatus and materials infected or suspected as being infected with TSE agent. In addition, the methods and compositions of the invention are suitably used in a prophylactic or precautionary mode, where definite knowledge of infection is uncertain. For example, the method of the invention can be easily incorporated into the standard sterilisation protocols used for preparation of surgical apparatus prior to use its use in surgical procedures.

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20 The methods and compositions of the present invention are also suitably used for the inactivation of TSE agents in potentially contaminated clinical waste and culled animal material. At present, this waste material is incinerated at 1000°C, which requires specialised facilities and is expensive. It is an advantage of the present invention that the TSE inactivation procedure can occur at temperatures and in conditions that do not require highly specialised facilities and the prospects of complete inactivation of the TSE agent are comparable to the more energy intensive and expensive incineration procedures.

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30 The proteolytic enzyme of the invention is typically a protease but can be suitably any biological polymeric molecule capable of catalysing cleavage of a polypeptide chain.

35 It is a feature of the invention that the proteolytic enzyme is a thermostable enzyme, that is, that it demonstrates optimal biological activity at temperatures in excess of the normal mammalian body temperature of 37°C. In embodiments of the invention the enzyme is thermally stable and biologically active, and inactivation is carried out, at temperatures equal to or greater than 40°C; preferably in the range of 50°C to 120°C; and more

preferably where the temperature is between 55 °C and 85 °C. In a specific embodiment of the invention the temperature is about 60 °C. In a further specific embodiment the temperature is about 80 °C.

5 Thermostable proteolytic enzymes suitable for use in the methods and compositions of the invention are obtainable from a number of sources such as thermophilic bacteria and archaea. In one embodiment of the present invention the thermostable proteolytic enzyme is isolated from thermophilic bacteria, hyperthermophilic bacteria and archaea. Suitable organisms for extraction of proteolytic enzymes include *Thermotoga maritima*; *Thermotoga neopolitana*; *Thermotoga thermarum*; *Fervidobacterium islandicum*; *Fervidobacterium nodosum*; *Fervidobacterium pennivorans*; *Thermosiphon africanus*; *Aeropyrum pernix*; *Thermus flavus*; *pyrococcus spp.*; *Sulfolobus solfataricus*; *Desulfurococcus*; *Bacillus thermoproteolyticus*; *Bacillus stearothermophilus*; *Bacillus sp. 11231*; *Bacillus sp. 11276*; *Bacillus sp. 11652*; *Bacillus sp. 12031*; *Thermus aquaticus*; *Thermus caldophilus*; *Thermus sp. 16132*; *Thermus sp. 15673*; and *Thermus sp. Rt41A*.

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20 It is apparent to a person of skill in the art that the aforementioned organisms are not the only sources of thermostable proteases. Indeed, some organisms that are not considered to be truly thermophilic can also express thermostable proteolytic enzymes. Such organisms are commonly termed thermodurable in that although they do not choose to live in conditions of high temperature, they can withstand high temperatures for limited periods. A number of *Bacillus* species fall in the category of thermodurability and are known to produce thermostable subtilisin-type proteases.

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30 The pH at which the inactivation is performed can range from acid to alkaline, but is typically in the region of pH 8-13, and preferably around pH 12. Similarly, the thermostable protease is active in a pH range from acid to alkaline, but typically is optimally active in the region of pH 8-13, and preferably around pH 12.

35 In an example of the invention in use, the proteolytic enzyme is extracted from a culture of the thermophilic bacteria or archaea. The culture is suitably maintained under the optimal conditions for the organism typically within a bioreactor. Thus, a continuous source of the organism can be maintained,

allowing proteolytic enzyme to be obtained whenever needed.

Alternatively, in an example of the invention in use described in more detail below, the gene encoding a thermostable proteolytic enzyme is isolated from the source organism, *Bacillus thermoproteolyticus*. The gene is used to generate a recombinant expression construct, typically a plasmid, which is transformed into a host organism, *Escherichia coli*. The transformed *E. coli* is grown in a bioreactor and when at an appropriate cell density the expression of the plasmid construct is initiated and the proteolytic enzyme harvested using standard methods. The recombinant expression route allows for the production of the proteolytic enzyme product under less extreme conditions of temperature than would be required for the original source organism.

The recombinant route is further advantageous in that it allows for the genetic manipulation of recombinant thermostable protease genes in order to increase thermal stability or biological activity or for some other purpose. Thus, the activity of a thermostable proteolytic enzyme can be readily optimised for use in the methods and compositions of the invention.

A second aspect of the invention provides for a method of sterilising apparatus, comprising the step of exposing the apparatus to a solution comprising a thermostable proteolytic enzyme.

The term "sterilising" is commonly understood to mean the procedure by which living organisms are removed from or killed in a substrate, such as a piece of equipment or a solution. In the present case the TSE agent, or prion, is not technically considered to be a living organism, in the sense that a bacterium or virus is, because it does not apparently contain any genetic material. However, the transmission of the TSE pathogenic agent between animals does result in disease. Thus, the term "sterilising" as used herein is applied to the procedure by which both pathogenic agents (such as TSE agents) and living organisms are removed from or killed in a substrate.

In a preferred embodiment, the method of the invention comprises maintaining the sterilising solution at a temperature below 100°C, and more preferably between 45°C and 85°C. The pH of the sterilising solution can range from acid to alkaline, but is typically in the region of pH 8-13, and preferably around

pH 12. Similarly, the thermostable protease is active in a pH range from acid to alkaline, but typically is optimally active in the region of pH 8-13, and preferably around pH 12.

5 In specific embodiments of the invention the sterilising solution is applied to the apparatus as a spray. The advantage of this mode of application is that, larger surface areas of apparatus, operating tables or even walls of rooms (for example in abattoirs) can be treated with the sterilising solution of the invention. Typically the solution will be heated to an optimal temperature, for example between 60°C to 80°C, before being sprayed onto the surface that is to be sterilised, that surface being optionally heated in advance.

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15 Alternatively, the apparatus is immersed in the sterilising solution for a predetermined period of time. Again, the temperature of the solution is typically optimised prior to immersion of the contaminated apparatus. It is optional to include ultrasonication means in the immersion bath to enable ultrasonic cleaning to occur at the same time as treatment with the sterilising solution of the invention.

20 In a third aspect, the invention provides for a method of sterilising material, comprising exposing said material to a first solution comprising a thermostable proteolytic enzyme; and then exposing the apparatus to at least a second solution comprising a second thermostable proteolytic enzyme. In use, the material is typically apparatus, surgical or meat rendering equipment, or TSE infected biological waste.

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30 By dividing the sterilisation method into at least two, and optionally more, successive steps the conditions in each step can be optimised to ensure maximum inactivation of any TSE agent present. Thus, the temperatures and/or pH of successive steps can be different. In specific embodiments of the invention the proteolytic enzymes in the first and second (and optionally more) solutions are the same, or are different.

35 In a fourth aspect the invention provides for a composition for inactivating a TSE agent, comprising a thermostable proteolytic enzyme.

Typically, the composition of the invention further comprises a buffering

agent. In a specific embodiment of the invention the buffering agent has a pK_a of between 8 and 13. Alkaline buffers suitable for use in the method of the invention include 4-cyclohexylamino-1-butanesulfonic acid (CABS) which has a pK_a of 10.7 at 25 °C, and 3-cyclohexylamino-1-propanesulfonic acid (CAPS) which has a pK_a of 10.4 at 25 °C.

5

Alternatively, the composition of the invention comprises sufficient sodium hydroxide or other alkaine agent to adjust the pH of the composition to alkaline, more preferably around pH 12. Addition of 1M sodium hydroxide to the composition of the invention, using a pH probe calibrated using Universal standards, is sufficient to set the pH of the composition to around 12.

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Further provided by the invention is apparatus for inactivating a TSE agent comprising:

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- a. a chamber for receiving contaminated material;
- b. means for controlling the temperature of the chamber; and
- c. a thermostable proteolytic enzyme active at alkaline pH, located within the chamber, the chamber optionally containing a solution of the thermostable proteolytic enzyme at a temperature of 45 °C to 85 °C.

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Further aspects of the invention provide for uses of the aforementioned compositions for the inactivation of TSE agents.

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An advantage of the invention is its use in degrading TSE and similar agents, and it has been found in operation of particular embodiments of the invention that TSE-contaminated material has been successfully decontaminated using a combination of elevated temperature of around 50 °C to 70 °C and alkaline pH of around 9 to 12, with a thermostable, alkophilic proteolytic enzyme. Whilst it is on occasion possible to achieve some decontamination by extremely high temperature alone, it is of significant benefit to be able to inactivate TSE whilst avoiding extreme conditions, such as extremes of temperature, which lead to damage to the equipment being decontaminated

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In a separate, though related, aspect of the present invention, the problem of detecting infective material is addressed. If it were possible to test an item of equipment for contamination either prior to or after carrying out a sterilisation process, then this would be of significant utility.

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An anti-prion antibody, mAb 6H4 is available commercially from Prionics, Switzerland. This antibody can be used to detect prion protein, detected typically using a second antibody conjugated to a detectable marker, which second antibody binds to the first.

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A difficulty that has been discovered by the present inventors is that binding of this antibody to equipment suspected of being contaminated with prion, or equipment that is suspected to be contaminated but which has been subjected to treatment intended to destroy the prion, does not correlate with infectivity.

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It has, for instance, been discovered by the inventors that prion-infected mouse brain homogenate, digested with protease, and run on SDS-PAGE, then probed with anti-prion antibody, shows a negative result, that is to say absence of antibody binding. This material nevertheless retains infectivity.

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A further object of the invention is to provide methods and reagents for identifying infective prion material and for determining when infective material has been removed by treatment.

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Accordingly, a further aspect of the invention provides a method of examining prion-infected or suspected prion-infected material and determining whether dimers of prion protein are present.

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This aspect of the invention is based upon a correlation between presence of dimers of prion protein and presence of infectivity. As the prion protein can exist in a number of different glycosylation states, references to dimer is intended to include dimers of the protein whether one or other or both is in any of a number of difference possible glycosylation states. Reference to dimer is also intended to include reference to dimers of fragments of prion protein, those fragment-containing dimers retaining infectivity. Proteolytic treatment of the prion can result in removal of part of the protein sequence, leaving behind a residue which in dimer form retains infectivity, and these fragment dimers are included within the definition of dimer in the present aspect of the invention.

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The method enables detection of presence of dimer and hence enables detection of presence of infective material in cases where previous tests, designed to identify whether the monomer is present, would have given a

negative result whereas in fact infectivity remained in that particular sample being tested. Hence, one advantage of the method is that at least some of the previous false negative results are eliminated. Avoiding these false negatives is clearly of significant value in testing alleged contaminated material as well as in testing material after treatment intended to remove infectivity.

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As set out in more detail in an example below, prion dimers can be detected by using an antibody that binds to a prion monomer, using a second antibody, which has been labelled, to bind to the anti-prion antibody, and determining whether a protein is identified having approximately twice the molecular weight of the expected molecular weight of the prion monomer. As mentioned, the prion can be glycosylated in a number of different positions, typically 0, 1 or 2 positions, so when western blotting is used, a number of bands may be seen on the resultant blot, corresponding to protein molecules having different glycosylation patterns. The monomer is expected to have a molecular weight of about 33 - 35 kDa at its full length, however, it is routinely observed on SDS-PAGE gels and immunoblots as a limited proteolytic cleavage product known as "PrP 27-30". Correspondingly, the dimer is expected to be found at that part of the blot corresponding to about 54 - 70 kDa.

The dimer forms may be probed using an antibody specific to the dimer, that is to say an antibody which binds to the dimer but substantially does not bind to the monomer form of the prion. This antibody may itself be labelled or may be probed using a secondary labelled antibody.

A preferred method of this aspect of the invention thus comprises a method of detecting prion infectivity comprising detecting prion dimer in a sample.

The invention additionally provides reagent for detecting prion dimers, and thus a further embodiment of the invention lies in an antibody specific for prion dimer, which antibody binds prion dimer but substantially does not bind prion monomer. This antibody optionally is labelled, and in an example described below the label is horseradish peroxidase; other suitable labels are alkaline phosphatase, beta galactosidase and D-biotin, though any suitable label may additionally be used.

An alternative reagent for detection of presence of dimer comprises an antibody that binds to both monomer and a dimer and a molecular weight standard that corresponds to the molecular weight of the dimer. These are suitably used in combination with, for example, Western blotting techniques, to identify a protein having a molecular weight approximately twice that of the prion monomer. All these reagents are suitably incorporated into prion dimer detection kits.

To make an antibody selective for prion dimer, an animal is immunised with prion dimer and then serum extracted from the animal is run on a prion dimer column to identify antibodies that bind the dimer. These are then tested for cross-reactivity with prion monomer, with cross-reacting antibodies removed. The removal can be effected using a column loaded with prion monomer, the antibodies that emerge therefrom then being tested for absence of cross-reactivity.

Isolated prion dimer forms a further embodiment of the invention. This isolated material can be obtained simply by cutting out a portion of the SDS-PAGE gel used to resolve prion dimers. Alternatively, other separation techniques can be used to extract the prion dimer from homogenised prion-infected mouse brain. Antibodies that bind to the prion monomer and which are cross reactive can be used to confirm that the material thus obtained is prion dimer and not other protein of the same molecular weight.

As set out in more detail in the examples, prion strain 301V, a mouse passaged isolate, derived from a Holstein-Fresian cow terminally ill with BSE is used as an example of a prion strain. Infection is known to be produced by intracerebral inoculation and the incubation period required for the onset of clinical symptoms is remarkably uniform. That is to say, providing that the dose of infectious agent is sufficient then the classic signs of disease will appear at a defined time post-inoculation (in the VM mice this is 120 days). For obvious reasons no-one has tested these properties on humans, however, the mouse bioassay is regarded as the closest available model and therefore a good indicator of BSE infection in man.

Using the Western blot detection system in an example below. the monomer is apparently completely removed by protease digestion at high pH and

temperature. Under these conditions, however, samples which were subsequently used *in vivo* did not prevent infection and may even have enhanced its onset. There must, therefore, be another source of infection other than the monomer. Since the monomer can be removed (or at worst dramatically reduced in concentration) this indicates a primary role in infectivity for the dimer - either alone or in combination with the monomer.

The invention thus also provides dimer removal using protease degradation which is optionally enhanced by environmental conditions - high temperature, extremes of pH, and/or the use of detergents (e.g. SDS). As well as using single enzyme treatments, combinations of proteases and/or other enzymes can be used. For example, better degradation of the infective agent may be achieved by the addition of lipases, peptidases, glycosylases, nucleases and other enzymes.

To confirm dimer removal, a dimer cross-reactive antibody can be used in conjunction with a suitable detection system, one example being a sensitive *in vitro* detection system currently available from Invitrogen, referred to as Western Breeze, to confirm removal prior to further mouse bioassays.

The methods and compositions of the inventions are described in more detail below and are illustrated by the accompanying drawings in which;

Fig. 1 shows the effect of temperature on a protease M digest of mouse brain homogenate (mbh);

Fig. 2 shows the effect of pH on a protease M digest of mbh;

Fig. 3 shows a *Bacillus thermoproteolyticus* Rokko digest of mbh;

Fig. 4 shows the effect of sodium dodecyl sulfate (SDS) on Rokko digest of mbh;

Fig. 5 shows an SDS-PAGE of mbh;

Fig. 6 shows an immunoblot of mbh;

Fig. 7 shows a protease G, R and C digest of mbh;

Fig. 8 shows an immunoblot of the digest in Fig. 7;

5 There is no figure 9; and

Fig.s 10 to 13 show blots as explained in the examples below.

EXAMPLES

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VM Mouse Colony and Incubation with BSE (301V) agent

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Studies on the inactivation of the TSE agent, BSE strain (301V), required the establishment of a mouse breeding colony for the generation of both uninfected and infected brain homogenate (mbh) and its subsequent titration and bioassay. The VM mouse strain selected for use in the study was obtained from Dr David Taylor (Institute of Animal Health, Edinburgh). Six pairs were introduced into a dedicated room within an animal facility. Mice were screened for their health status and a breeding programme initiated.

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BSE (301V) infectious mouse brain (IAH, Edinburgh) was prepared for inoculation by crude homogenisation followed by passage of the brains through increasingly fine gauge luer-locked needles (from 21G-27G) to and from a contained safety syringe into a closed septum-topped vial. This procedure was carried out in a validated safety cabinet within a containment level 3 (CL3) laboratory immediately prior to intracerebral inoculation of the VM mice. The anaesthetised (alphadolone/alphaxalone) mice were inoculated intracerebrally via 26 gauge needles with 20ml of the mouse brain homogenate preparation. Forty-nine out of fifty mice survived this procedure. These were retained to allow incubation of the agent and the generation of the required quantity of high-titre infectious material.

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Biomass Production

35

A wide variety of organisms were chosen for the production of biomass in order to provide as broad a selection of thermostable proteolytic enzymes as possible. Organisms selected ranged from those growing optimally at

moderately thermophilic temperatures (50°C) through to extremely thermophilic temperatures (100°C) and included members of both the Archaea and the Bacteria. Thermophiles were also selected to cover a wide range of growth pH, encompassing pH optima from pH2.5 to pH11.5. The majority of organisms were grown in batch culture, however, where the growth requirements of the organism were particularly fastidious, continuous culture was used (Raven and Sharp (1997) Applied Microbial Physiology: A Practical Approach, Ch. 2, Eds. Stanbury and Rhodes, OUP pp.23-52). Depending on the biomass yield of the organism being grown, batch culture volumes of between 20L and 120L were employed to achieve the desired amount of cell paste. The continuous culture system utilised either a 2L or 5L working volume gas lift bioreactor constructed entirely of glass and PTFE. More prolific organisms such as *Bacillus* spp., and *Thermus* spp., were pre-screened to select those with high levels of protease activity prior to their culture on a larger scale. A quick and sensitive fluorometric protease assay utilising microtitre plates was adopted for this purpose to permit high through-put screening (EnzChek™, Molecular Probes, Leiden, Netherlands).

Culture biomass was harvested by continuous centrifugation (Contifuge Stratos™, Kendro Laboratory Products, Bishop Stortford, UK) and stored at -80°C. Culture supernatants were concentrated with a 10KDa cut off tangential flow filter (Pall filtration, Portsmouth, UK). Proteins were precipitated with ammonium sulphate (90% saturation) and stored at -80°C.

25 Protein Purification

A rapid protease screening and purification technique was required in order to process all of the crude protein preparations after the biomass production stage. A dye-ligand affinity chromatography system was used for this purpose (PIKSI M™, Affinity Chromatography Ltd., Isle of Man, UK). Initially, each crude ammonium sulphate precipitate was dissolved in buffer and passed through a desalting column. Each sample was then loaded onto the PIKSI M test kit, which contained 10 different affinity ligands. Fractions were then assayed for protease activity to determine the most suitable matrix for purification of the protease, either by positive binding of the target molecule and then elution, or by negative binding of contaminants. The purification was then scaled up using the same affinity matrix in conjunction with an FPLC

system (Amersham-Pharmacia Biotech, Amersham, UK). By combining this technique with the fluorometric protease assay, the rapid screening of many fractions could be undertaken.

5 The fluorometric protease assay utilises casein derivatives that are heavily labelled with green fluorescent BODIPY FL in which the conjugates' fluorescence is almost totally quenched. Protease catalysed hydrolysis releases the highly fluorescent label and the resultant fluorescence can be measured on a fluorometric microplate reader (Labsystems Fluoroscan IITM).
10 The increase in fluorescence is proportional to protease activity and was compared with that of a standard protease (Protease X, Sigma-Aldrich, Poole, UK).

Protease Characterisation

15 A range of thermostable proteases were analysed (see Table 1). Direct characterisation of activity was carried out using the closest non-infectious analogue to BSE (301V) - infectious mouse brain homogenate available as substrate, i.e. normal VM mouse brain homogenate. Initial digests of total uninfected mouse brain homogenate (mbh) were performed over thirty minutes
20 at 60°C and at pH7.0. The samples were then boiled under reducing conditions and analysed by SDS-PAGE on pre-cast NuPage 4 - 12% Bis-Tris gels (NovexTM, San Diego, US). Gels were fixed using standard procedures and the proteins visualised using the Novex colloidal blue staining kit. The results showed both the levels of activity of the proteases against the mbh substrate under these conditions and also the level of purity of individual proteases. Protease concentrations used in the assays were based on total protein concentration, therefore, a wide range of activities was observed from limited to complete digestion.
25
30 Activity profiles showing the full effect of pH and temperature on mbh digestion were then produced for each enzyme. Several enzymes showed extensive activity over the complete range of conditions (pH 2 - 12 and 50 - 100°C). In general, however, complete digestion of mbh was only achieved over a narrower range indicative of the enzymes' pH and temperature optima.
35 Figs. 1 and 2 show the activity profiles for a sample protease, *Bacillus* protease M. As can be seen the enzyme is moderately thermophilic and gives

complete digestion at temperatures up to 70°C. Similarly the pH profile indicates a preference for neutral or alkaline conditions.

Table 1:

	Organism	Domain	Growth	Topt	pH opt
1	<i>Aeropyrum pernix</i>	Archaeon	Aerobe	95°C	7.0
2	<i>Alicyclobacillus acidocaldarius</i>	Bacterium	Aerobe	65°C	3.5
3	<i>Archaeoglobus fulgidus</i>	Archaeon	Anaerobe	85°C	6.5
4	<i>Bacillus caldotenax</i> BT1	Bacterium	Aerobe	65°C	7.0
5	<i>Bacillus pallidus</i>	Bacterium	Aerobe	65°C	9.0
6	<i>Bacillus stearothermophilus</i> L32-65	Bacterium	Aerobe	65°C	7.0
7	<i>Bacillus stearothermophilus</i> LUDA T57	Bacterium	Aerobe	65°C	7.0
8	<i>Bacillus thermoproteolyticus</i> Rokko	Bacterium	Aerobe	65°C	7.0
9	<i>Bacillus</i> sp. 11231	Bacterium	Aerobe	65°C	7.0
10	<i>Bacillus</i> sp. 11276	Bacterium	Aerobe	65°C	7.0
11	<i>Bacillus</i> sp. 11652	Bacterium	Aerobe	65°C	7.0
12	<i>Bacillus</i> sp. 12031	Bacterium	Aerobe	65°C	7.0
13	<i>Desulfurococcus</i> sp.	Archaeon	Anaerobe	85°C	6.5
14	<i>Fervidobacterium pennivorans</i>	Bacterium	Anaerobe	70°C	8.5
15	<i>Hyperthermus butylicus</i>	Archaeon	Anaerobe	95°C	6.5
16	<i>Pyrococcus furiosus</i>	Archaeon	Anaerobe	95°C	7.5
17	<i>Pyrococcus horikoshii</i>	Archaeon	Anaerobe	95°C	7.0
18	<i>Sulfolobus acidocaldarius</i> 98-3	Archaeon	Aerobe	75°C	2.5
19	<i>Sulfolobus hakonensis</i>	Archaeon	Aerobe	75°C	2.5
20	<i>Sulfolobus solfataricus</i> P1	Archaeon	Aerobe	75°C	2.5
21	<i>Sulfolobus solfataricus</i> P2	Archaeon	Aerobe	75°C	2.5
22	<i>Thermobrachium celere</i>	Bacterium	Anaerobe	65°C	8.5
23	<i>Thermococcus fumicola</i> s	Archaeon	Anaerobe	85°C	6.5
24	<i>Thermus caldophilus</i> GK24	Bacterium	Aerobe	70°C	8.0
25	<i>Thermus aquaticus</i> YT1	Bacterium	Aerobe	70°C	8.0
26	<i>Thermus</i> sp. 16132	Bacterium	Aerobe	70°C	8.0
27	<i>Thermus</i> sp. 15673	Bacterium	Aerobe	70°C	8.0
28	<i>Thermus</i> sp. Rt41A	Bacterium	Aerobe	70°C	8.0

Protease Testing

Several enzymes did not give complete digestion of mbh even when incubation times were increased to 24 hours. This may have simply been due to low protease activity, however, several highly purified and concentrated enzymes still only produced partial digests. In these reactions little enzyme-substrate interaction appeared to be occurring. It was considered that this could be due to non-proteinaceous material, e.g. lipids, surrounding the substrate and preventing interaction, however, pre-treatment with lipases and other enzymes had no observable effect (data not shown). A second possibility considered was the effect of repulsive surface charges between proteases and the substrate. One enzyme in particular, purified from *Bacillus thermoproteolyticus*

Rokko, consistently produced poor digestion of mbh even at high concentrations. This enzyme was known to remain active in the presence of high levels of detergents, therefore, digests were performed with the addition of SDS in an attempt to overcome this effect. Figures 3 and 4 show 5 *B.thermoproteolyticus* Rokko digests of mbh in the absence and presence of SDS. Figure 3 clearly indicates that under standard conditions there is little difference in digestion even as the protease concentration is increased to 20mg.ml⁻¹. On addition of SDS (lanes 2-5), no protein bands, except the protease itself, are visible indicating a complete digest. Tested proteases could, 10 therefore, be divided simply into three categories: (i) those able to give total mbh digestion in the absence of detergent, (ii) those able to give total mbh digestion in the presence of SDS and (iii) those unable to digest the substrate fully. Proteases in categories (i) and (ii) were selected for immediate further study, while those in category (iii) were either rejected or assumed to be 15 required in greater quantity and/or higher purity.

Western Blotting of Mouse Brain Homogenate

Mbh proteins were transferred onto nitrocellulose and blocked overnight in PBS- 20 Tween (PBST) + 3% skimmed milk powder. The membrane was washed (x3 in PBST) and incubated for 1 hour with 6H4 anti-human recombinant PrP monoclonal antibody (Prionics, Zurich, Switzerland). After a second washing step, anti-mouse HRP-conjugate was added and the membrane incubated for 1 hour. Washing was repeated and the antibody reaction visualised by addition of TMB (Harlow and Lane 25 (1988), Antibodies: A Laboratory Manual, Cold Spring Harbor Press).

Figures 5 and 6 show an SDS-PAGE and an immunoblot of undigested mbh respectively. Although there is some non-specific background, dark bands indicating the presence of mouse prion can clearly be seen at the expected 30 molecular weight (~33-35kDa). Distinct bands are also visible at ~66-70kDa, which may correspond to prion dimers previously reported (Safar *et al.* (1990) Proc. Natl. Acad. Sci. USA, 87:pp6373-6377).

Western transfer and immunoblotting were used to confirm that no 35 immunoreactive fragment of mouse PrP^c remained after digestion. Figure 7 shows mbh digested to completion with three closely related thermostable

proteolytic enzymes (Proteases G, R and C), as assessed by SDS-PAGE. In the corresponding immunoblot (Figure 8) only 2 bands are visible, both with an apparent MW of ~23kDa. The strong band in lane 9 corresponds to recombinant mouse PrP (+ve control), whilst the weak band in lane 4 appears 5 to be due to a slight reaction with the heavily loaded enzyme preparation. In this case, complete loss of PrP^c immunoreactivity is still apparent since no band is observable in lane 3.

Preparation and Titration of Infectious Mouse Brain Homogenate

10

Eighty days post challenge onward, mice were subject to daily clinical scoring to detect clinically affected mice as early as possible. A single mouse died of an unknown cause prior to this period. The remainder exhibited the expected disease progression and were sacrificed between 110 and 130 days post 15 challenge. The brains were removed aseptically and stored frozen until required. Forty-eight BSE (301V) infectious VM mouse brains were homogenised in four volumes of PBS within a contained homogeniser then passed sequentially through increasingly fine gauge needles (21G to 26G) until free flowing. A sample with a further 2-fold dilution (1:9 mouse brain: PBS) 20 was prepared for titration of infectivity. Over 800 x 0.1ml aliquots of BSE (301V) infectious mouse brain homogenate were prepared. These procedures were again carried out under rigorous class III containment, including the wearing of positive pressure respirators.

25 Groups of 25 eight week old VM mice received titration doses of the infectious mouse brain homogenate preparation at 10 fold dilutions from 10^{-1} – 10^{-8} . A further group of 25 mice were challenged with uninfected mouse brain homogenate as a control. All mice were inoculated under anaesthetic using 26G x 3/8" needles with plastic sleeve guards cut off 2mm below bevel in a 30 Class 2 cabinet with the use of an injection guard. The mice were then left to incubate the BSE (301V) agent for extended periods some in excess of a year. The initial titre of the infectious mouse brain homogenate preparation was established retrospectively once all incubations (clinical monitoring at 80 days onwards) were complete.

Dimer detection in digest d mouse brain

BSE (301V)-infected mouse brain homogenate was digested at neutral pH and 60°C for 30 minutes with protease. Total protein digests were run on SDS-

5 PAGE and transferred by Western blotting to nitro-cellulose membranes. These were cut into strips and probed with CAMR anti-prion antibodies (produced in rabbits). A second generic antibody (goat anti-rabbit) was conjugated to horseradish peroxidase and used with detection by TMB colorimetric substrate.

10 At the time, the expected result was that the results were the same as the control blot (number 7). - using the anti-prion antibody mAb 6H4 (from Prionics, Switzerland). In this control blot, there is seen the typical three-banded pattern (glycosylation states) for protease-digested infectious-conformation prion protein (PrP^{Sc}).

15 However, the blots in this example did not show this pattern. Blot 1 uses a polyclonal antibody raised against a PPD-conjugated peptide corresponding to an N-terminal region of the prion molecule. Nothing is seen in the lanes. This section of the protein is susceptible to proteolysis, so it is not surprising to see 20 nothing in the lanes (2&3) – see figure 10, blot 1 on left hand side. Lane 1 is a molecular weight marker.

25 Blot 2 has a second antibody raised against a peptide sequence further into the prion molecule. This shows at least 9 bands of varying intensity, approximately equidistant, at a molecular weight corresponding to a prion dimer with a range of glycosylation states – see blot 2 on figure 10.

Blot 3 antibody shows similar profile; blot 4 is also shown but its results are too poor quality to draw any conclusions – see blots 3 and 4 on figure 10.

30 Blots 5 and 6, shown on figure 11 with the control blot 7, again show the multi-banded pattern

Dimer detection in digested m use brain

35 The above example was repeated, and the results shown in figures 12 and 13.

Blot 1 shows molecular weight markers in lanes 1 and 5. Lane 2 is recombinant murine PrP showing recombinant murine PrP oligomers. Lane 3 shows lack of antibody response to protease-digested infectious mouse brain homogenate. Lane 4 is the antibody response in the undigested control.

5

Blot 2 is as above but shows the previous banding pattern in the protease digested sample.

Blot 3 shows the antibody 3 response. Here there is some response to 10 recombinant murine PrP (lane 2). Lane 3 shows not only the multiple (dimeric PrP) banding pattern, but also some monomeric PrP response.

Blot 7 is the 6H4 mAb antibody control. Here there is good detection of recombinant murine PrP oligomers (lane 2). Lane 3 shows the heavily 15 diglycosylated form of limitedly protease-treated PrP^{Sc}, plus the more minor monoglycosylated and non-glycosylated forms typical of BSE (301V) strain. No 'dimer' detection is apparent.

Preparation of antibodies including dimer preferential antibody

20

In the examples, we have used 6 polyclonal antibodies Of these, three detect the dimer alone and do not bind the monomer whereas one cross-reacts with both the monomer and the dimer.

25 The polyclonal sera were produced by immunisation of rabbits with synthesised prion mimetic peptides. These peptides were designed based on regions of high homology between human, mouse and bovine prion protein amino acid sequences.

30 The sequences producing the dimer-reactive antibodies were as follows:

CGGWGQPHGGC (Peptide2)

CGGYMLGSAMSRPIIHFGNNDYEC (Peptide 3)

CVNITIKQHTVTTTKEENFTETDC (Peptide 5)

35 CITQYQRESQAYYQRGASC (Peptide 6)

The peptides were synthesised with a cysteine at both ends (see above) and with a cysteine at one end only. This method was used in order to present both the linear form and a loop structure of the antigen on the surface of the carrier protein.

5

The peptides were synthesised commercially and coupled to the carrier protein PPD (purified protein derivative), derived from an attenuated strain of the bacterium *Mycobacterium bovis*, which is lyophilised and used to conjugate to the peptide via a linker.

10

Anti-prion polyclonal antibodies were produced as follows:

2. A sample of pre-immune sera (~1ml) was collected from each of a group of Dutch rabbits.

15 3. The rabbits were injected with reconstituted freeze-dried Bacillus Calmette-Guerin (BCG) vaccine for intradermal use. A dose of 0.1ml of reconstituted BCG vaccine was given in two sites in the scruff of the neck of the rabbit.

20 4. After 4 weeks, 0.6mg of each peptide-PPD conjugate was measured (0.3mg of each of the 1 cysteine and 2 cysteine versions) and dissolved in 1ml of sterile 0.9% saline.

25 5. An equal volume of incomplete Freunds adjuvant was added and 0.75ml aliquots, of the resulting emulsion, were injected intra-muscularly into each hind limb and 0.25ml aliquots into two sites in the scruff of the neck per rabbit.

30 6. After 4 weeks a boost injection was given comprising of the peptide-PPD conjugates prepared as in step 3 and 4. The boost injections consist of four 0.25ml injections into the scruff of the neck of each rabbit.

7. 7-14 days after the first boost injections, 4ml test bleeds were taken, the sera was assessed by ELISA for antibody titre.

35 8. A second boost injection was given 4-6 weeks after the first.

9. A third boost injection given 4-6 weeks later.

10. A 4ml test bleed was taken 6-8 weeks after the third boost injection and antibody titres determined by ELISA

35 11. A fourth boost injection given.

12. A 4ml test bleed was taken 7-14 days after the fourth boost injection and antibody titre determined by ELISA.
13. Terminal exsanguination was carried out and blood collected. The serum was separated by centrifugation and stored at -20°C.

5

Analysis of antibody titre was achieved using ELISA. The immunoassay plate was coated with the same peptides conjugated to a different carrier protein (KLH) in order to differentiate the response to the peptide from the response to the carrier protein.

10

Three of the antibodies produced by immunisation of the synthetic peptide sequences described bind preferentially to the dimer form of the molecule.

Analagous steps may also be used to prepare a monoclonal antibody. This could
15 be achieved using a method such as described in Antibodies – A Laboratory Manual, Ed Harlow and David Lane, 1988 (Cold Spring Harbor Laboratory).

CLAIMS

1. A method for inactivating a transmissible spongiform encephalopathy (TSE) agent comprising exposing the TSE agent to a thermostable proteolytic enzyme.
2. A method according to claim 1, comprising exposing the TSE agent to the thermostable protease at a temperature that is equal to or greater than 40°C.
3. A method according to claim 2, wherein the temperature is between 50°C and 120°C.
4. A method according to claim 3, wherein the temperature is between 55 °C and 85 °C.
5. A method according to claim 4, wherein the temperature is about 60 °C.
6. A method according to claim 4, wherein the temperature is about 80 °C.
7. A method according to any previous claim wherein the pH is neutral (pH 7.0).
8. A method according to claims 1-6, wherein the pH is acidic.
9. A method according to claims 1-6, wherein the pH is alkaline.
10. A method according to claim 9, wherein the pH is between 8 and 13.
11. A method according to claim 9, wherein the pH is about 12.
12. A method according to any previous claim wherein the TSE agent is a prion.
13. A method according to any previous claim, wherein the TSE agent is

selected from the group consisting of Creutzfeld-Jacob disease; variant Creutzfeld-Jacob disease; Kuru; fatal familial insomnia; Gerstmann-Straussler-Scheinker syndrome; bovine spongiform encephalopathy; scrapie; feline spongiform encephalopathy; chronic wasting disease; and transmissible mink encephalopathy.

14. A method according to any previous claim wherein the thermostable proteolytic enzyme is obtained from a thermophilic organism.
15. A method according to claim 14 wherein the thermophilic organism is selected from the group consisting of archaea; hyperthermophilic bacteria and thermophilic bacteria.
16. A method according to claim 15 wherein the thermophilic organism is selected from the group consisting of *Thermotoga maritima*; *Thermotoga neopolitana*; *Thermotoga thermarum*; *Fervidobacterium islandicum*; *Fervidobacterium nodosum*; *Fervidobacterium pennivorans*; *Thermosiphon africanus*; *Aeropyrum pernix*; *Thermus flavus*; *pyrococcus* spp.; *Sulfolobus solfataricus*; *Desulfurococcus*; *Bacillus thermoproteolyticus*; *Bacillus stearo-thermophilus*; *Bacillus* sp. 11231; *Bacillus* sp. 11276; *Bacillus* sp. 11652; *Bacillus* sp. 12031; *Thermus aquaticus*; *Thermus caldophilus*; *Thermus* sp. 16132; *Thermus* sp. 15673; and *Thermus* sp. Rt41A.
17. A method of sterilising apparatus comprising exposing said apparatus to a solution comprising a thermostable proteolytic enzyme.
18. A method according to claim 17, wherein the solution is maintained at a temperature below 100°C.
19. A method according to claim 17, wherein the solution is maintained at a temperature of between 45°C and 85°C.
20. A method according to any of claims 17-19, wherein the solution has a neutral pH.

21. A method according to any of claims 17-19, wherein the solution has an acidic pH.
22. A method according to any of claims 17-19, wherein the solution has an alkaline pH.
23. A method according to claim 22, wherein the solution has a pH of between 8 and 13.
24. A method according to any of claims 17-23, wherein the thermostable proteolytic enzyme is obtained from a thermophilic organism.
25. A method according to claim 24 wherein the thermophilic organism is selected from the group consisting of archaea; hyperthermophilic bacteria and thermophilic bacteria.
26. A method according to any of claims 17-25, wherein the solution is applied to the apparatus as a spray.
27. A method according to any of claims 17-25, wherein the apparatus is immersed in the solution.
28. A method of sterilising apparatus, comprising exposing said apparatus to a first solution comprising a thermostable proteolytic enzyme; and then exposing the apparatus to at least a second solution comprising a second thermostable proteolytic enzyme.
29. A method according to claim 28, wherein the first and second proteolytic enzymes are the same.
30. A method according to claim 28, wherein the first proteolytic enzyme is different to the second proteolytic enzyme.
31. A method according to any of claims 28-30, wherein the pH of the first solution is different to the pH of the second solution.

32. A method according to any of claims 28-31, wherein the temperature of the first solution is different to the temperature of the second solution.
33. A composition for inactivating a TSE agent, comprising a thermostable proteolytic enzyme.
34. A composition according to claim 33, further comprising a buffering agent.
35. A composition according to claim 34, wherein the buffering agent has a pK_a of between 8 and 13.
36. A composition according to claim 33, further comprising sufficient sodium hydroxide to set the pH of the composition to around 12.
37. A composition according to claims 33-36, further comprising a detergent compound.
38. A composition according to claim 37, wherein the detergent is sodium dodecyl sulphate (SDS).
39. A composition according to any of claims 33-38, wherein the thermostable proteolytic enzyme is obtained from a thermophilic organism.
40. A composition according to claim 39 wherein the thermophilic organism is selected from the group consisting of archaea; hyperthermophilic bacteria and thermophilic bacteria.
41. Use of a composition according to claims 33-40 for sterilising material contaminated with a TSE agent.
42. Apparatus for inactivating a TSE agent comprising:
 - a. a chamber for receiving contaminated material;

- b. means for controlling the temperature of the chamber; and
- c. a thermostable proteolytic enzyme active at alkaline pH, located within the chamber.

43. Apparatus according to claim 42 comprising a solution of the thermostable proteolytic enzyme at a temperature of 45 °C to 85 °C.

44. A method of examining a sample infected with or suspected to be infected by prion protein, comprising detecting dimers of prion protein in the sample.

45. A method according to Claim 43 comprising determining whether the sample contains prion monomer, prion dimer, or mixtures of prion monomer and dimer.

46. A method according to Claim 44 or 45, comprising determining whether the sample contains a dimer of a fragment of a prion.

47. A method according to any of Claims 44 to 46, comprising probing the sample with an antibody specific to prion monomer, and determining whether the sample contains protein with a molecular weight corresponding to approximately twice that of prion monomer.

48. A method according to any of Claims 44 to 46, comprising probing the sample with an antibody specific to prion dimer.

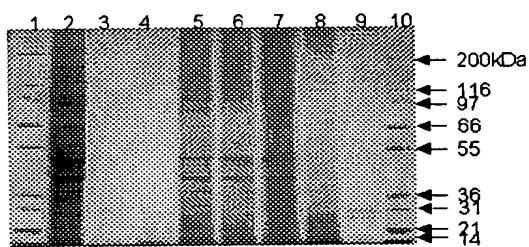
49. A method of detecting prion infectivity, comprising detecting prion dimer in a sample.

50. An antibody, which is specific for prion dimer but does not bind to prion monomer.

51. A labelled antibody according to Claim 50.

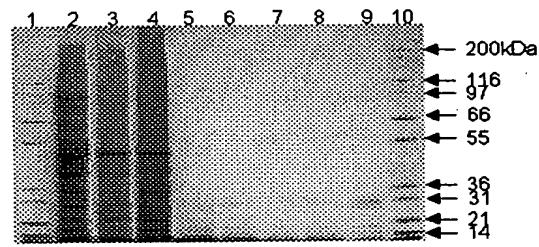
52. A method of producing an antibody, comprising immunising an animal with a prion dimer, obtaining an extract therefrom which contains antibodies, and isolating from said extract antibodies which bind prion dimer.
53. A method according to Claim 52, comprising obtaining an antibody preparation containing antibodies which bind prion dimer, and removing from said preparation antibodies which also bind prion monomer.
54. Purified prion dimer.

Fig. 1



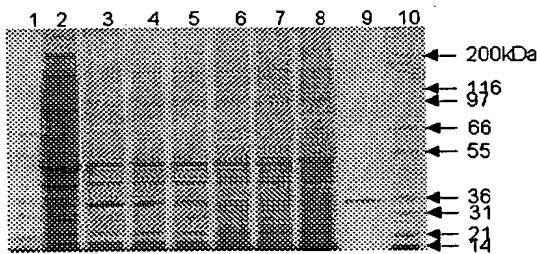
Lanes 1 & 10, marker proteins; lane 2 untreated mbh; lane 3, 50°C; lane 4, 60°C; lane 5, 70°C; lane 6, 80°C; lane 7, 90°C; lane 8, 100°C; lane 9, Protease M.

Fig. 2



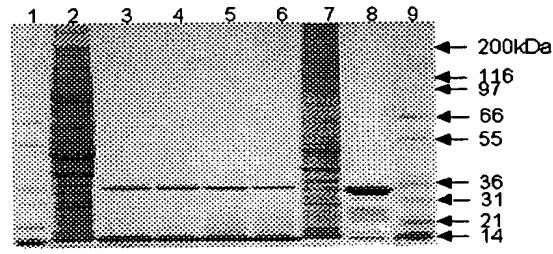
Lanes 1 & 10, marker proteins; lane 2 untreated mbh; lane 3, pH2; lane 4, pH4; lane 5, pH6; lane 6, pH8; lane 7, pH10; lane 8, pH12; lane 9, Protease M.

Fig. 3



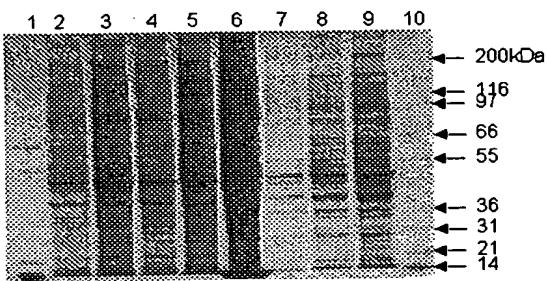
Lanes 1 & 10, marker proteins;
lane 2 untreated mbh; lanes 3 - 8,
Rokko digest (20mg.ml^{-1} -
 0.1mg.ml^{-1}), lane 9, Rokko
(1mg.ml^{-1}).

Fig. 4



Lanes 1 & 9, marker proteins; lane 2
untreated mbh; lane 3, 2% SDS; lane
4, 1% SDS; lane 5, 0.5% SDS; lane 6,
0.25% SDS; lane 7, mbh + 2% SDS;
lane 8, Rokko (20mg.ml^{-1}).

Fig. 5



Lanes 1 & 10, marker proteins; lanes 2 & 3, mbh; lanes 4 - 6, mbh pellet; lanes 7 - 9, mbh supernatant.

Fig. 6

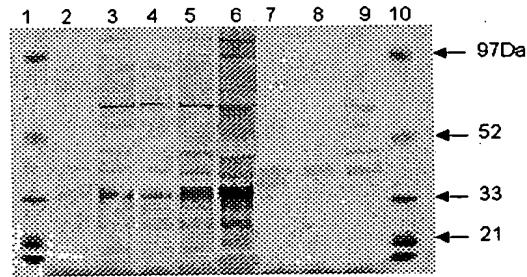
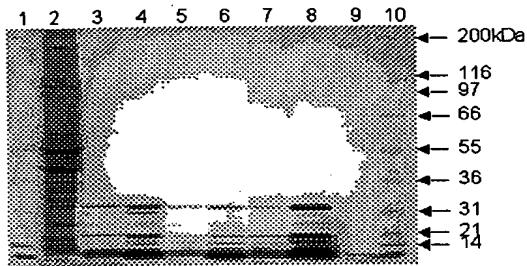
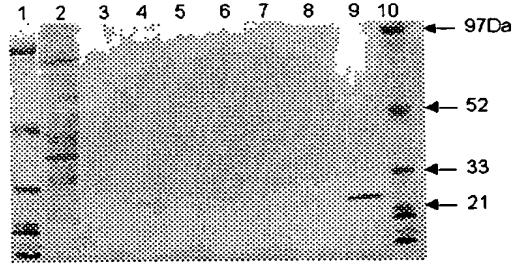


Fig.7



Lanes 1 & 10, marker proteins; lane 2, untreated mbh; lane 3, Protease G digest; lane 4, Protease G; lane 5, Protease R digest; lane 6, Protease R; lane 7, Protease C digest; lane 8, Protease C; lane 9, rec. mouse PrP.

Fig.8



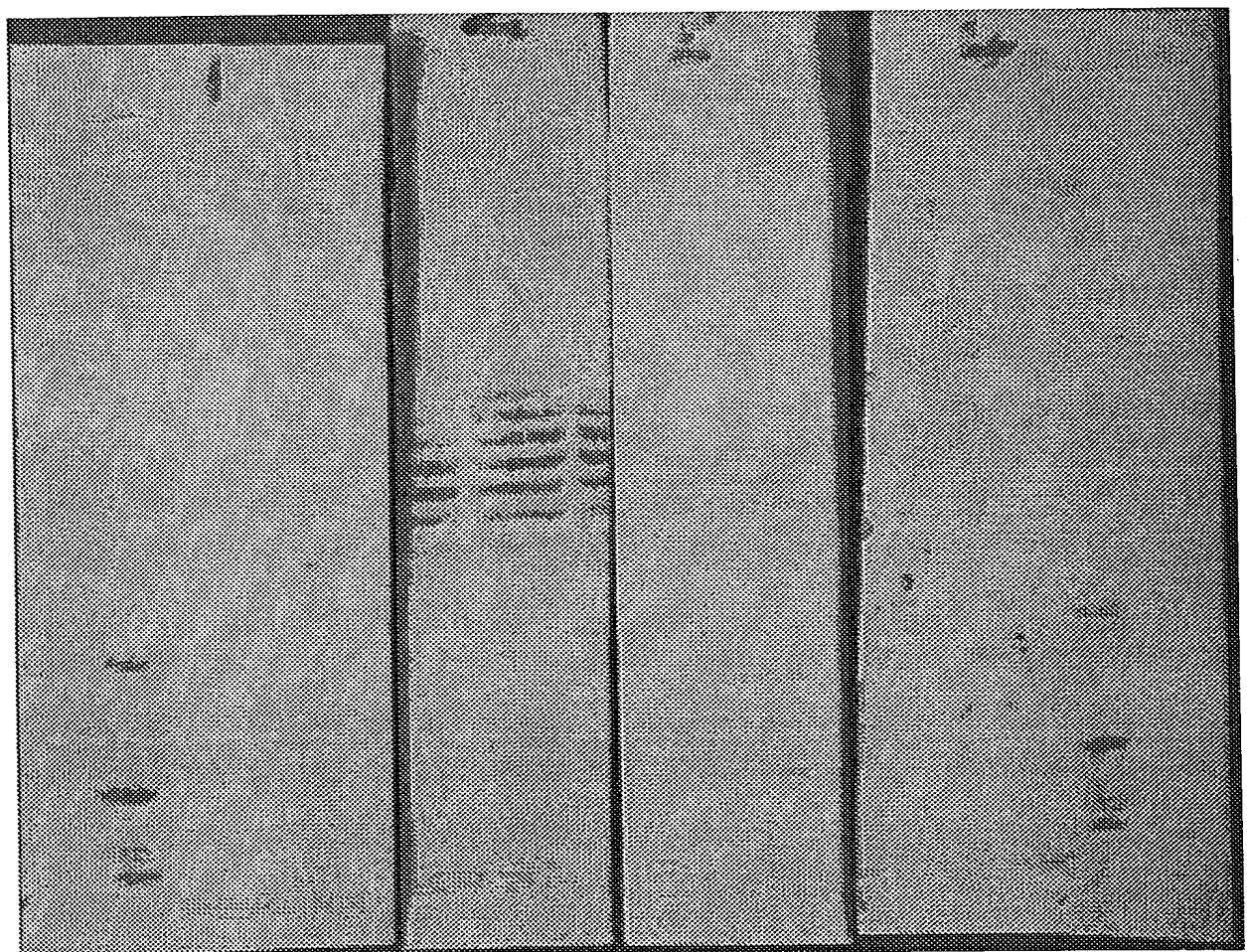


Fig. 10



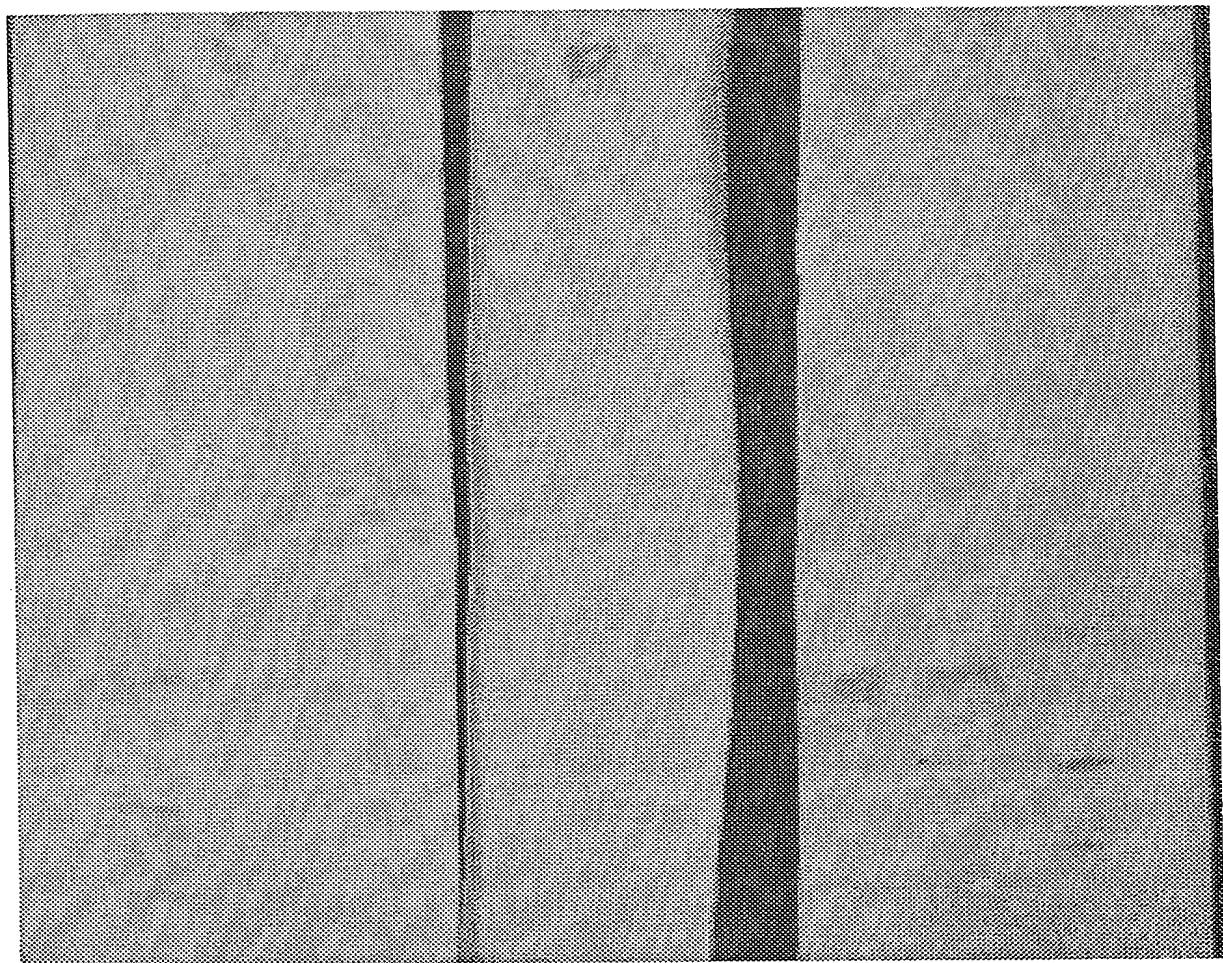


Fig. 11

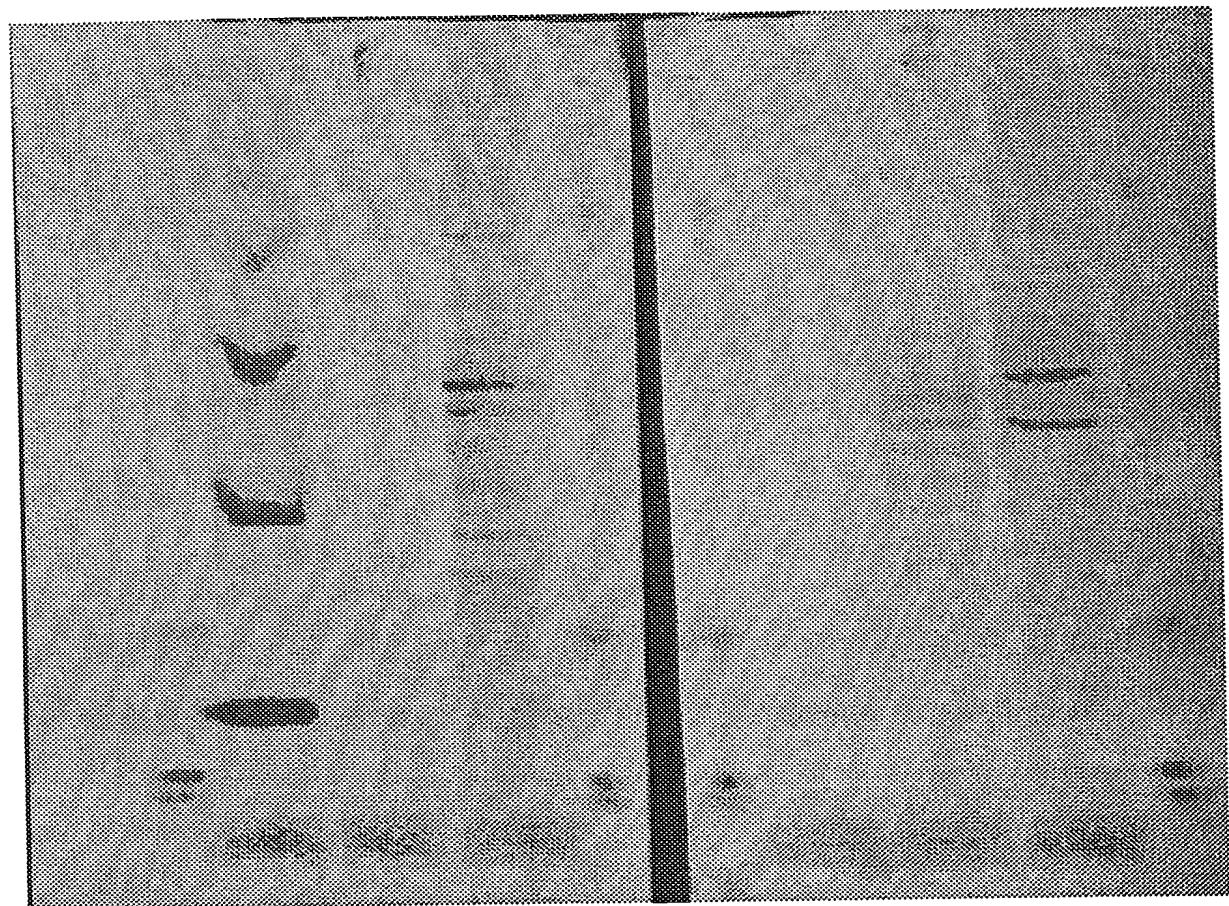


Fig. 12

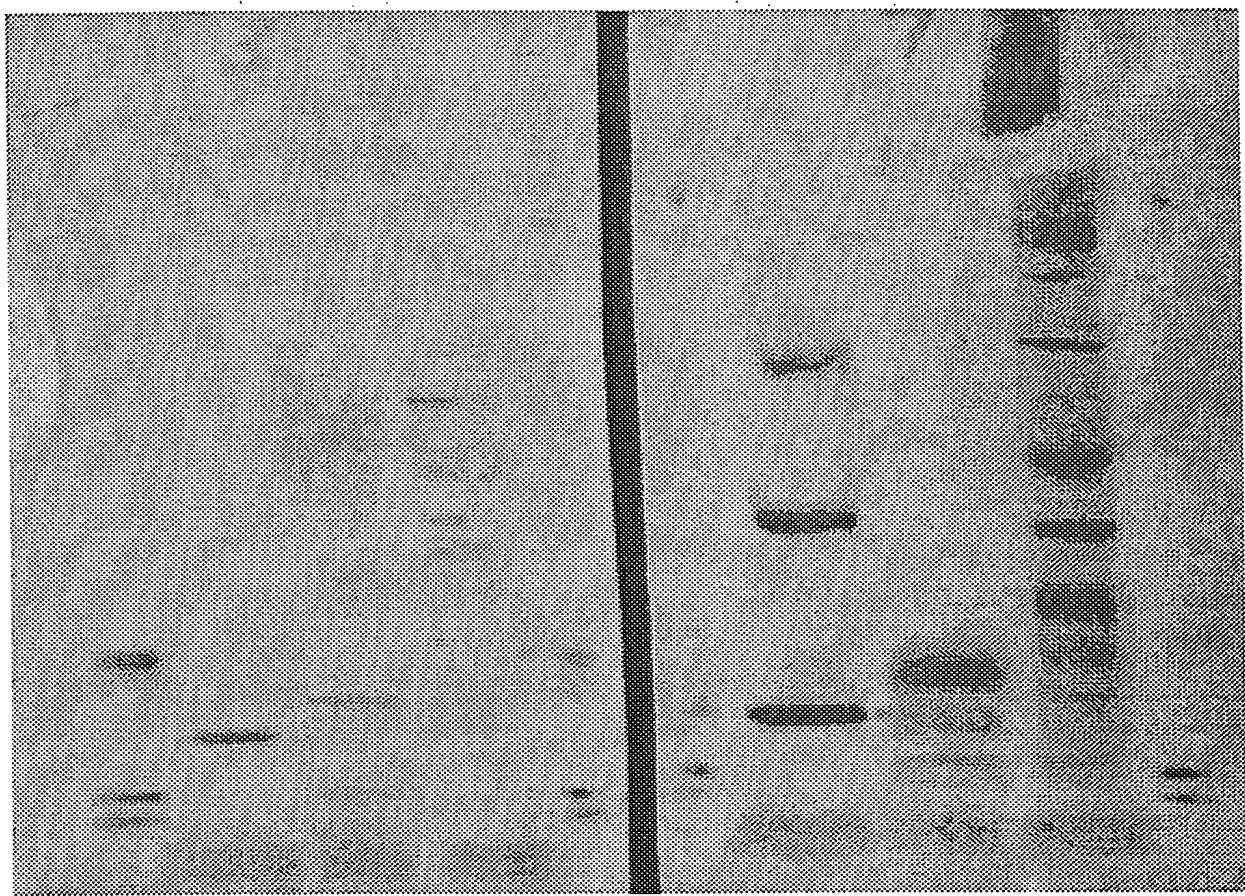


Fig. 13

